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## A new class of NO-donor pro-drugs triggered by $\gamma$ -glutamyl transpeptidase with potential for reno-selective vasodilatation†

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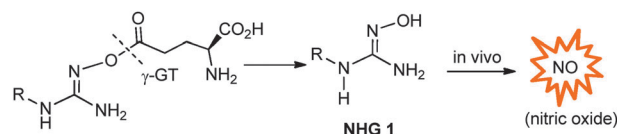
**This communication describes the synthesis of a new class of *N*-hydroxyguanidine (NHG) pro-drugs which release nitric oxide (NO), triggered by the action of  $\gamma$ -glutamyl transpeptidase ( $\gamma$ -GT), and have potential for the treatment of acute renal injury/failure (ARI/ARF).**

Acute renal injury (AKI), or failure (ARF), is a common complication that affects millions of people worldwide, particularly in intensive care units, where it is associated with a mortality rate of between 50% and 80%.<sup>1</sup> There is no effective pharmaceutical therapy to date. One of the major causes of AKI is ischemia-reperfusion injury,<sup>2,3</sup> following aortic ring cross-clamping during by-pass surgery, which can lead to renal ischemia.<sup>4</sup> Reperfusion of ischemic renal tissue causes the generation of reactive oxygen species which induce renal cell injury<sup>5</sup> and promote impairment of renal perfusion at least in part *via* inactivation of the vasodilator, nitric oxide (NO).<sup>6–8</sup> Thus, a kidney selective vasodilator with antioxidant properties is attractive to maintain blood flow to offset AKI and scavenge the reactive oxygen species. Localisation of activity to the kidney would avoid a systemic reduction in blood pressure. Dopamine and fenoldopam, specific agonists of the dopamine-1 receptor, have been used clinically in an effort to reduce the risk of perioperative renal dysfunction, but the effectiveness of these agents is not clear.<sup>9,10</sup> We hypothesised that an effective exogenous NO-donor, which selectively increases renal vasodilatation, would offer an alternative.

There are a wide range of NO-donor drugs in existence,<sup>11</sup> including conventional organic nitrates and nitrites, *S*-nitrothiols, NONOates and *N*-hydroxyguanidines (NHGs).<sup>12–16</sup> The NHGs **1** are analogues of *N*<sup>o</sup>-hydroxy-L-arginine (NOHA), a biosynthetic intermediate involved in the generation of NO from L-arginine.<sup>11</sup> Several enzymatically activated NHG pro-drugs have been reported such as peptidylglycine  $\alpha$ -amidating monooxygenase (PAM)-active *O*-carboxymethyl *N*-hydroxyguanidines<sup>17</sup> and *N*- $\beta$ -galactosidases-active ( $\beta$ -D-galactopyranos-1-yl)oxyguanidine.<sup>18</sup> Our approach aimed to mask the NO generating *N*-OH group with a  $\gamma$ -glutamyl residue to facilitate activation by the enzyme,  $\gamma$ -glutamyl transpeptidase ( $\gamma$ -GT). Given that  $\gamma$ -GT is primarily expressed in the kidney (5–10 fold higher than in the liver and pancreas),<sup>19</sup> it was envisaged that this enzyme could be used to trigger reno-selective release of an NHG and subsequent *in situ* generation of NO (Scheme 1). A similar strategy has been described for reno-selective L-3,4-dihydroxyphenylalanine (L-DOPA), the Glu-DOPA.<sup>20,21</sup>

However, the direct coupling of NHGs with a  $\gamma$ -glutamyl residue was hampered by intramolecular cyclization and dehydration leading to a 1,2,4-oxadiazole ring; or alternatively lactamization and release of a pyroglutamic acid (Scheme 2, data not included).

In an effort to prevent these modes of cyclization, we investigated the use of a bridge between the NHG and the  $\gamma$ -glutamyl group. Both  $\gamma$ -glutamyl itself and  $\gamma$ -aminobutanoyl (GABA)<sup>22</sup> were explored as linkers. Thus **2a** and **2b** became synthesis targets (Scheme 3) and they were prepared *via* appropriately protected dipeptide intermediates (ESI;† Scheme S1). Unfortunately **2a** gradually decomposed presumably due to the carboxylic acid moieties promoting autodegradation.

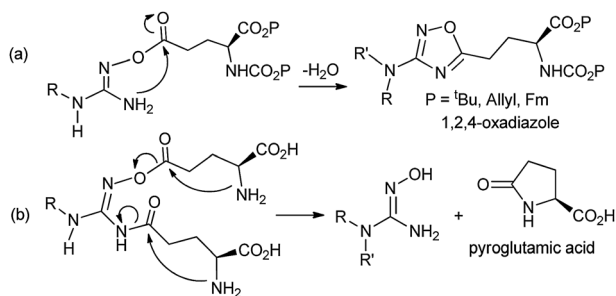


**Scheme 1** Approach to  $\gamma$ -GT triggered release of NHG **1** and the reno-selective release of nitric oxide.

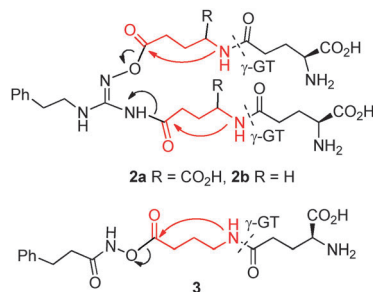
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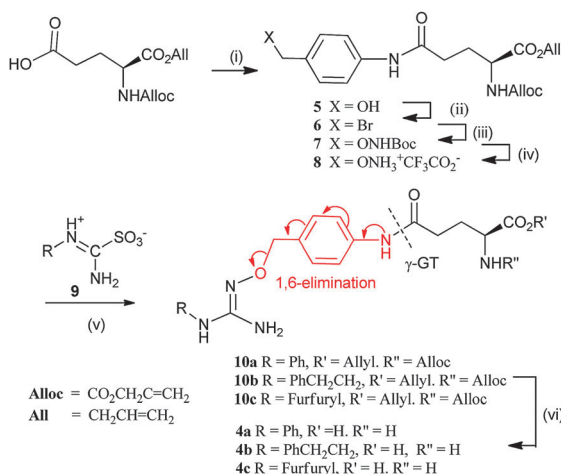


**Scheme 2** Cyclization of direct coupling of NHGs with  $\gamma$ -glutamyl residue(s).



**Scheme 3** Design of Glu/Gaba linked  $\gamma$ -glutamyl NO-donor pro-drugs of NHG and hydroxamic acid.

On the other hand, **2b** could be purified by preparative HPLC but was found to be resistant to  $\gamma$ -GT-mediated cleavage *in vitro* and was considered not to be a useful pro-drug. This prompted the preparation of **3** (Scheme 3), involving the conjugation of only one GABA-Glu dipeptide onto a hydroxamic acid, an alternative NO-donor.<sup>11</sup> Compound **3** too, unfortunately, was found to be resistant to  $\gamma$ -GT mediated deacylation, suggesting that the GABA-Glu peptide linker is not suitable for  $\gamma$ -GT cleavage in this setting.



**Scheme 4** Design and synthesis of aminobenzyl linked  $\gamma$ -glutamyl NO-donor pro-drugs of NHG: (i) 4-aminobenzylalcohol, EEDQ, DCM, rt, 12 h, 85%; (ii)  $\text{PBr}_3$ , THF, 0 °C, 2 h, 87%; (iii)  $\text{BocNHOH}$ , NaH, THF, 0 °C, 4 h, 83%; (iv)  $\text{CF}_3\text{CO}_2\text{H}$ , DCM, 92%; (v) **9a** R = Ph or **9b** R =  $\text{PhCH}_2\text{CH}_2$  or **9c** R = furfuryl,  $\text{Et}_3\text{N}$ , DMAP, DCM, 38–53%; (vi)  $[\text{Pd}(\text{PPh}_3)_4]$ ,  $\text{PhSiH}_3$ , DCM, 37–89%.

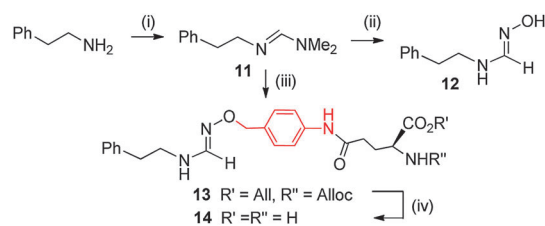
$\gamma$ -Glutamyl anilines are known substrates for  $\gamma$ -GT<sup>23</sup> and presented an alternative linker option. The success of such an approach would involve a 1,6-elimination following the action of  $\gamma$ -GT on *N*- $\gamma$ -glutamylaminobenzyl-oxo-guanidine **4a–c**, as illustrated in Scheme 4. Similar spacers have been employed previously in anticancer pro-drug design.<sup>24</sup>

In the event, the synthesis of **4a–c** was successfully accomplished through a six-step reaction sequence (Scheme 4). Firstly,  $\gamma$ -glutamylation of 4-aminobenzylalcohol with Alloc- $\gamma$ -glutamic acid 1-allyl ester (Alloc-Glu-OAll) (ESI;† Scheme S1) gave benzyl alcohol **5**. Conversion of the benzylalcohol moiety to the corresponding bromide **6** followed by nucleophilic displacement with  $\text{BocNHOH}$  generated aminooxide **7**, and then treatment with  $\text{CF}_3\text{COOH}$ -DCM, gave the key intermediate **8** which was coupled with the required amino(alkyl/aryl)iminio-methanesulfonate **9a–c** to generate **10a–c**. Finally the All/Alloc groups were removed under neutral conditions with  $[\text{Pd}(\text{PPh}_3)_4]/\text{PhSiH}_3$  to give **4a–c**.

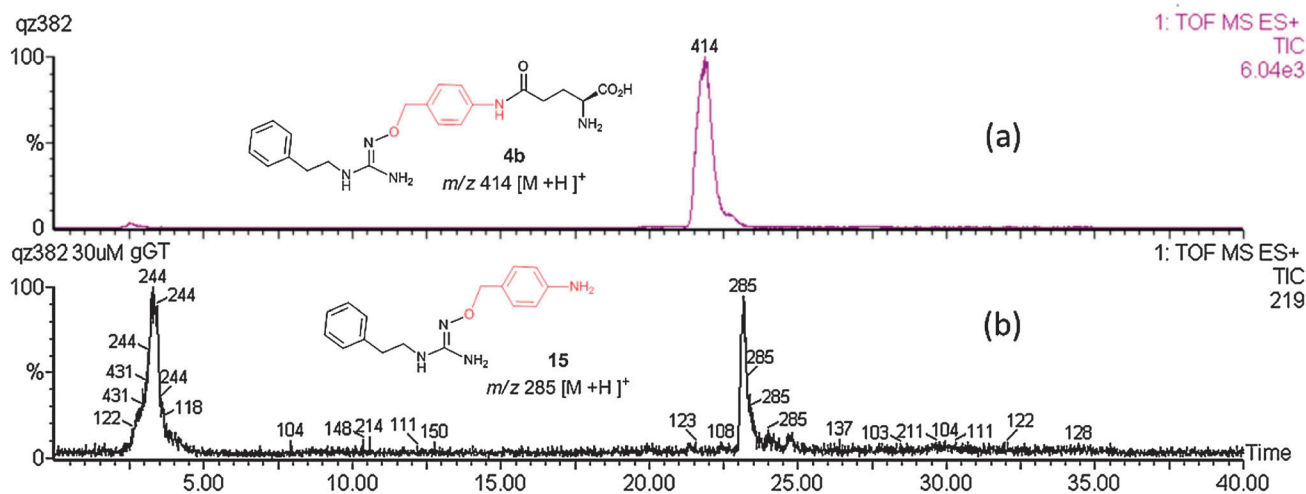
The same aminobenzyl linker was also used for the  $\gamma$ -glutamylation of *N*-hydroxyformamides (NHF) (Scheme 5). *N*-Hydroxy-*N*-(4-butyl-2-methylphenyl)formamide<sup>25</sup> and *N*-hydroxy-*N*-(3-chloro-4-morpholin-4-ylphenyl)formamide<sup>26</sup> have been documented as 20-hydroxyeicosatetraenoic acid (20-HETE) inhibitors. 20-HETE is a major metabolite of arachidonic acid and is a potent vasoconstrictor; localisation of an NHF would counter the effect of 20-HETE and induce a synergic vasodilation effect mediated by NO. Thus *N*'-hydroxyphenylethylformamide **12** was prepared in this study and converted to pro-drug **14**.

Pro-drugs **4a–c** and **14** were rapidly cleaved by  $\gamma$ -GT and they were completely deacylated after 1 h, as judged by LC-MS. Fig. 1(a) and (b) illustrates the LCMS trace of **4b** and the conversion of **4b** to deacylated intermediate **15**  $[\text{M-Glu}]^+$  by  $\gamma$ -GT. This was in clear contrast to the GABA-linked candidates **2b** and **3**, which proved to be resistant to the action of  $\gamma$ -GT. 1,6-Elimination and loss of the linker from **15** to generate the parent NHG **1b** is significantly slower (trace amount of parent **1b** was detected by selective ion monitoring at  $m/z$  180) than the cleavage of the  $\gamma$ -glutamyl moiety. In preliminary experiments with animal tissue, LC-MS analysis revealed ~90% conversion of **4b** (100  $\mu\text{M}$ ) to **1b** in a rat renal homogenate (37 °C; 45 min). In addition, **4b** was found to induce substantial vasodilation in rat isolated perfused kidney preparations (50% of maximum vasodilation induced by ~40  $\mu\text{M}$  **4b**). Details of the bioactivity of these pro-drugs will be reported elsewhere.

In summary, several candidate NO-donor pro-drugs have been prepared, designed for activation by  $\gamma$ -GT. The pro-drugs



**Scheme 5** Synthesis of *N*-hydroxyformamide and its glutamyl pro-drug: (i)  $\text{Me}_2\text{NCH}(\text{OMe})_2$ , reflux, 2 h, quantitative; (ii)  $\text{NH}_2\text{OH}\cdot\text{HCl}$ , MeOH, 63%; (iii) **8**, THF, reflux, 29%; (iv)  $[\text{Pd}(\text{PPh}_3)_4]$ ,  $\text{PhSiH}_3$ , DCM, rt, 6 h, 53%.



**Fig. 1** LCMS trace of **4b** incubated in Krebs buffer at 37 °C for 1 h (a) without  $\gamma$ -GT and glutamyl acceptor Gly-gly, **4b** is intact; (b) with  $\gamma$ -GT (100 mU mL<sup>-1</sup>) and glutamyl acceptor Gly-gly (5 mM), **4b** is deglutamylated to give the species **15**.

comprise the parent NO-donor, a linker and a  $\gamma$ -glutamyl moiety. GABA-linked pro-drugs are not suitable substrates for  $\gamma$ -GT, but those linked by the aminobenzyl moiety proved to be good substrates for the enzyme. The  $\gamma$ -glutamyl group is cleaved rapidly, with a slower decomposition of the aminobenzyl linker. Improved design is now focussed on tuning the spacer to encourage a more rapid release of the parent NHG drug.

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